Regulation of Cyclin D3 by Calpain Protease in Human Breast Carcinoma MDA-MB-231 Cells

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The Ca^{2+}-activated neutral protease calpain induced proteolysis has been suggested to play a role in certain cell growth regulatory proteins. Cyclin proteolysis is essential for cell cycle progression. D-type cyclins, which form an assembly with cyclin-dependent kinases (cdk4 and cdk6), are synthesized earlier in G1 of the cell cycle and seem to be induced in response to external signals that promote entry into the cell cycle. Here we show that cyclin D3 protein levels are regulated at the posttranscriptional level by calpain protease. Treatment of human breast carcinoma MDA-MB-231 cells with lovastatin and actinomycin D resulted in a loss of cyclin D3 protein that was completely reversible by the peptide aldehyde calpain inhibitor, LLnL. The specific inhibitor of the 26S proteasome, lactacystin, the lysosome inhibitors, ammonium chloride and chloroquine, and the serine protease inhibitor, phenylmethylsulfonylfluoride (PMSF), did not block the degradation of cyclin D3 by lovastatin and actinomycin D. Results of *in vitro* degradation of cyclin D3 by purified calpain showed that cyclin D3 protein is degraded in a Ca^{2+}-dependent manner, and the half-life of cyclin D3 protein was dramatically increased in LLnL treated cells. These data suggested that cyclin D3 protein is regulated by the Ca^{2+}-activated protease calpain.

Key words – Cyclin D3, proteolysis, calpain

Introduction

Cell cycle transitions in eukaryotic cells occur through activation and inactivation of cyclin-dependent kinases (cdks). Cdks become activated or inactivated by complexing with their specific cyclins and cdk inhibitors which are expressed during the cell cycle[4]. The major mammalian G1 cyclins include three D-type cyclins (cyclin D1, D2 and D3) and cyclin E. D-type cyclins are synthesized earlier in G1 phase and seem to be induced in response to external agents that promote entry into S phase of the cell cycle by complexing to cdk4 and cdk6. They also promote progression through a G1 checkpoint by phosphorylating the retinoblastoma protein (pRB), and can contribute to oncogenesis via their deregulated expression achieved through gene amplification, chromosomal rearrangement, or retroviral integration[11,23].

There is an evidence that intracellular proteases play important roles in cell growth control, although the underlying mechanisms are still poorly understand. Regulated proteolysis of specific proteins including cell cycle control proteins, oncoproteins, tumor suppressors and transcription factors has been shown to be critical for proper control of cell function[5,14]. In this regard, two proteolytic systems have been studied: the ubiquitin-proteasome pathway and Ca^{2+}-dependent protease calpain. The ubiquitin pathway plays an important role in the degradation of many short-lived regulatory proteins. Degradation of a protein via this system involves two successive steps, conjugation of multiple molecules of ubiquitin to the target protein and degradation of the tagged molecule by the 26S proteasome[1,25]. Accumulating data indicate that *in vivo* and *in vitro* substrates for ubiquitin include cell cycle control proteins, oncoproteins, tumor suppressors, and transcription factors[9,25]. The neutral cysteine protease calpains (EC 3.4.22.17) represent the other major class of nonlysosomal proteases functioning in a Ca^{2+}-dependent fashion[7,32,33]. Until now, calpain was thought to be activated as a cellular receptor in response to calcium mobilization, not only in physiolsoical states but also in various pathological conditions. Therefore, calpain is regarded as a biomodulator acting through limited proteolysis of relevant substrates proteins, including cytoskeletal and membrane

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proteins, enzymes such as kinase and phosphatases, and transcription factors[27]. Solomon and Goldberg[28] reported that the proteasome can degrade monomeric (free) myofibrillar proteins, except when they are associated with other myofibrillar proteins. These data suggest that another protease must exist upstream of the proteasome to provide it with monomeric myofibrillar proteins as substrate. Interestingly, accumulating experimental evidence implicates both the ubiquitin proteasome pathway and calpain in the degradation of the tumor suppressor and transcription factor, such as p53, YY1, AP-1, N-myc, and it was proposed that the two pathways may play a role in targeting the protein under various conditions[8,10,15,17,34]. Several studies point to the involvement of the proteasome in cell cycle regulatory proteins degradation such as cyclin A, B and E [2,6,14,22] however, it is not clear whether proteasome is the key rate-limiting protease or whether it is simply one protease in the pathway of degradation of cyclins. Previously, we have identified that calpain has a potential role in the degradation of cyclin D1 protein[3].

In the present study, we have investigated the biochemistry of the proteolytic mechanism that leads to loss of cyclin D3 protein in human breast carcinoma cells. Using inhibitors for various proteolytic systems we show that degradation of cyclin D3 protein involves the Ca^{2+}-activated protease calpain. We have also demonstrated an increased half-life of cyclin D3 protein by inhibition of calpain activity, clearly suggesting that cyclin D3 function is regulated by Ca^{2+}-dependent protease calapin.

Materials and Methods

Cell culture

The human breast carcinoma MDA-MB-231 cells were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were maintained as a monolayer culture in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, penicillin and streptomycin (Biofluids, Rockville, MD, USA) at 36°C in 5% CO_2.

Protease inhibitors and reagents

Calpain inhibitors, LLnL (calpains inhibitor I, N acethyl-leucyl-leucyl-norleucinal) and LLM (calpains inhibitor II, leucyl-leucyl methionial), serine protease inhibitor phenylmethylsulfonylfluoride (PMSF), lysosome inhibitors (ammonium chloride and chloroquine), and purified m-calpain were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Lactacystin, a specific 26S proteasome inhibitor, was obtained from Oncogene Research Products (Cambridge, MA, USA). Actinomycin D and cychloheximide were purchased from Sigma.

Immunoblot analysis

Western blot analysis was performed as described previously[3]. Briefly, cells were washed in phosphate-buffered saline (PBS), and lysed with TNE buffer at 4°C for 20 min. Protein concentration was determined with the BIO-RAD protein assay (Bio-Rad Laboratories, Hercules, CA, USA), following the procedure described by the manufacture. Equal amounts of protein were subjected to electrophoresis on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA) by electroblotting. After extensive washing with TNE buffer, the blots were incubated with rabbit polyclonal anti-D-type cyclin 3 antibody or mouse monoclonal anti-cyclin B1 antibody (Santa Cruz Biotechnology,Inc.,Santa Cruz, CA, USA), and incubated with peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin (Amersham Life Science, Arlington Heights, IL, USA). The proteins were visualized with an enhanced chemilunescence (ECL) detection system (Amersham).

Degradation of cyclin D3 by calpain

To assess *in vitro* degradation of cyclin D3 by purified calpain, purified m-calpain was incubated with cell lysates for 1 h at 30°C as described previously[3]. After incubation SDS sample buffer was added, the samples were boiled, centrifuged, and the supernatant was used for Western blot analysis.

Results

Effect of calpain inhibitors on the loss of cyclin D3 protein in lovastatin and actinomycin D treated cells

We used lovastatin and actinomycin D as tools to examine the mechanisms governing the proteolysis of cyclin D3. Lovastatin is an inhibitor of 3 hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), an enzyme required for the conversion of HMG-CoA to mevalonic acid in the pathway leading to cholesterol synthesis[12] that has been used to induces a reversible G1 arrest in a

variety of cell types[16]. When human breast carcinoma MDA-MB-231 cells were incubated with lovastatin, steady state levels of cyclin D3 protein were markedly decreased and by 36 h the cyclin D3 protein levels were undetectable (Fig. 1A, lane 4). Actinomycin D, the RNA synthesis inhibitor, also induced the rapid loss of cyclin D3 protein (Fig. 1B, lane 4). To determine whether protease inhibitors could prevent the degradation of cyclin D3 in lovastatin and actinomycin D-treated cells, we first compared the effects of the peptide aldehyde calpain inhibitors, LLnL and LLM, and the serine protease inhibitor, PMSF, on cyclin D3 levels in log phase untreated control, and lovastatin and actinomycin D-treated cells. As shown in Fig. 1, LLnL completely prevented the loss of cyclin D3 protein (Fig. 1A and B, lane 5), but PMSF (Fig. 1A and B, lane 6) and LLM (data not shown) had no effect.

Lactacystin prevents the degradation of cyclin B1 but not cyclin D3

We next compared the effect of LLnL and lactacystin, a specific 26S proteasome inhibitor, on the loss of cyclin D3 and cyclin B1 protein in lovastatin and actinomycin D-treated MDA-MB-231 cells. As shown in Fig. 2, the steady-state levels of cyclin B1 protein were also markedly decreased in cells exposed to lovastatin and actinomycin D for 36 h and 24 h (Fig. 3A and B, lane 3), respectively. LLnL, an inhibitor of both calpain and the proteasome, blocked degradation of both cyclin B1 and cyclin D3 protein. In contrast, lactacystin prevented the loss of cyclin B1, and not cyclin D3 protein. Treatment of cells with lovastatin and actinomycin D also induced loss of cyclin A proteins that was reversible by lactacystin (data not shown). These data are consistent with previous reports showing that cyclin B1 degradation is through the proteasome[6,13], also they further support a calpain-mediated, non-proteasomal degradation mechanism for regulation of cyclin D3 under these experimental conditions.

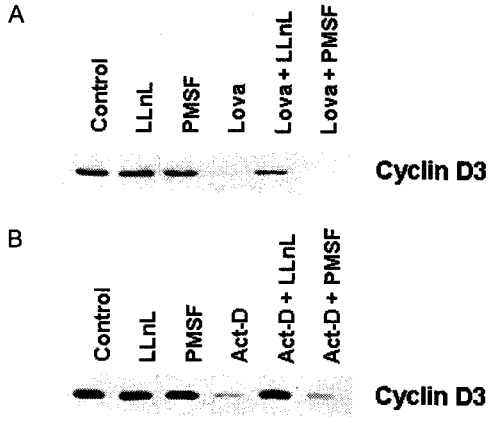

Fig. 1. Effect of calpain inhibitor LLnL on the levels of cyclin D3 protein in lovastatin and actinomycin D-treated MDA-MB-231 cells. (A) Cells were treated with lovastatin (Lova., 10 μM) for 36 h. After incubation with lovastatin for 24 h, LLnL (100 μM) or PMSF (1 mM) was added. (B) Cells were treated with actinomycin D (Act-D, 500 ng/ml) for 24 h, in the presence or absence of LLnL or PMSF for the final 12 h. For determination of cyclin D3 protein levels, equal amounts of cell lysate (30 μg protein) were resolved by 10% SDS-polyacrylamide gel electrophoresis and subjected to Western blot analysis using polyclonal anti-cyclin D3 antibody and ECL detection system.

Lack on effects of cystein protease and lysosome inhibitors on the loss of cyclin D3 protein

The effect of the lysosome inhibitors, ammonium chloride and chloroquine, on the levels of cyclin D3 protein in lovastatin-treated MDA-MB-231 cells was also investigated. Cells were incubated with or without lovastatin for 36 h, respectively, and then the lysosome inhibitors were added to the culture dishes for the last 12 h of incubation. Cyclin D3 protein levels of cells treated with the lysosome inhibitors were similar to those of untreated control cells

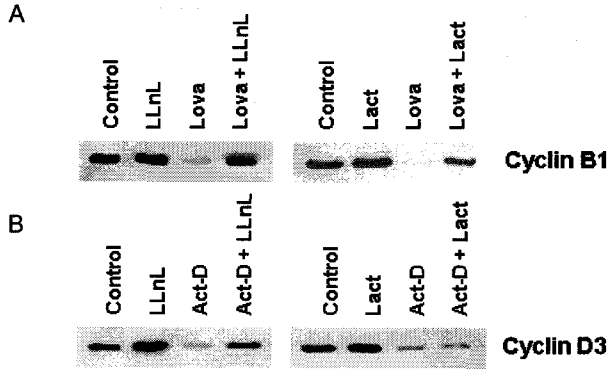

Fig. 2. Comparative effect of LLnL and lactacystin treatment on the levels of cyclin B1 and cyclin D3 protein in lovastatin and actinomycin D-treated MDA-MB-231 cells. (A) Cells were treated with LLnL or lactacystin (Lacta, 10 μM) for 12 h or lovastatin for 36 h. After incubation with lovastatin for 24 h, LLnL or lactacystin was added. (B) Cells were treated with actinomycin D for 24 h, in the presence or absence of LLnL or lactacystin for the final 12 h. The levels of cyclin B1 and cyclin D3 protein were determined by Western blot analysis using monoclonal anti-cyclin B1 and polyclonal anti-cyclin D3 antibody.

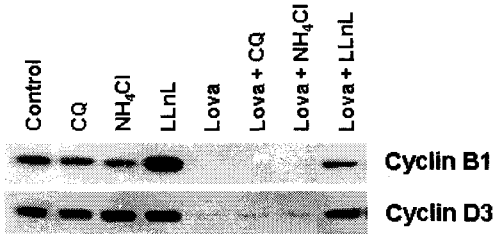

Fig. 3. Comparative effect of lysosome inhibitors on the levels of cyclin B1 and cyclin D3 protein in control and lovastatin D-treated MDA-MB-231 cells. Untreated control cells were compared to cells treated with chloroquine (CQ, 100 μM) and ammonium chloride (NH_4Cl, 2.5 mM) for 12 h, cells treated with lovastatin for 36 h or cells treated with lovastatin for 36 h and CQ or NH_4Cl for the last 12 h. The levels of cyclin B1 and cyclin D3 (B) protein were determined by Western blot analysis using monoclonal anti-cyclin B1 and polyclonal anti-cyclin D3 antibody.

(Fig. 3, lanes, 2 and 3). However they did not block the lovastatin-induced loss of cyclin D3 (Fig. 3, lanes, 6 and 7). These data further indicate that the proteolysis of cyclin D3 is specifically calpain associated.

In vitro degradation of cyclin D3 by calpain

Since our results above suggested that calpain may act as an endogenous regulator of cyclin D3 protein, we examined whether cyclin D3 acts as a substrate for purified calpain. Calpain, the Ca^{2+}-activated neutral endopeptidase, is an intracellular nonlysosomal cystein protease that requires calcium ions for its activity[21,32]. In the absence of added $CaCl_2$ resulted in no detectable loss of cyclin D3 protein (Fig. 4, lane 2). Boiling of calpain before addition also resulted in no detectable loss of cyclin D3 (Fig. 4, lane 4). Incubation of cell lysates with calpain and increasing concentration of $CaCl_2$ caused a striking concentration-dependent increase in the magnitude of calpain activity between 0.1 to 0.5 mM (data not shown). In the presence of 6.0 mM $CaCl_2$, calpain caused a complete loss of detectable cyclin D3 (Fig. 4, lane 3). Similarly, incubation of cell lysates with calpain and the calcium chelator EGTA caused a complete loss of calpain activity toward cyclin D3 protein. Thus cyclin D3 is a substrate for Ca^{2+}-dependence calpain-mediated proteolysis.

Stability of cyclin D3 protein increases in response to LLnL

If cyclin D3 is degraded by calpain *in vivo*, then inhibition of calpain in cells would be expected to stabilize

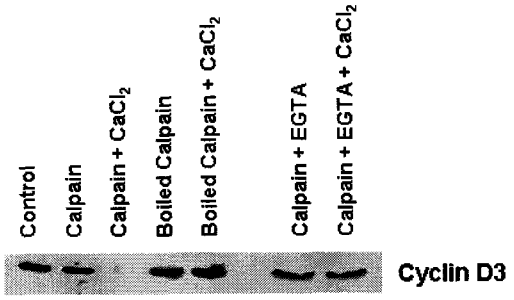

Fig. 4. *In vitro* degradation of cyclin D3 by purified calpain in MDA MB-231 cells. Cell lysates from MDA-MB-231 cells were incubated for 1 h at 30°C with purified calpain or boiled-calpain in the presence or absence of 6 mM $CaCl_2$ or 10 mM calcium chelator EGTA. For determination of cyclin D3 protein levels, equal amounts of cell lysate were used for Western blot analysis.

cyclin D3 protein. The half-life of cyclin D3 protein in the presence or absence of LLnL was therefore determined in MDA-MB-231 cells. To determine cyclin D3 half-life, 60-70% confluent monolayer of control cells or cells treated with LLnL for 12 h were treated with the protein synthesis inhibitor cycloheximide. At each time point indicated in Fig. 5, cells were washed with cold PBS, lysed in lysis buffer, and cyclin D3 levels were determined by Western blot

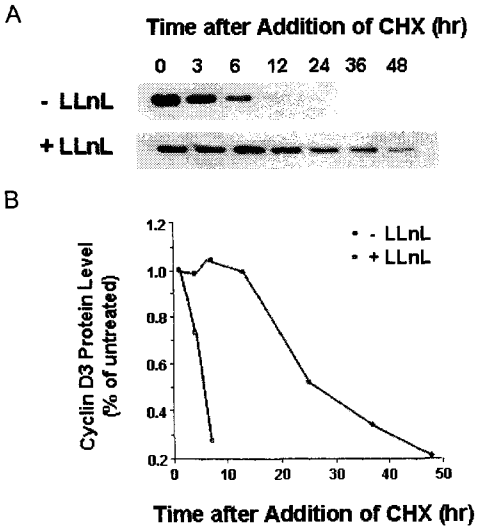

Fig. 5. Calpain inhibition leads to stabilization of cyclin D3 protein in MDA-MB-231 cells. (A) Cells were grown in the absence or presence of LLnL for 12 h and then cycloheximide (CHX, 100 μg/ml) was added for the times indicated, and the levels of cyclin D3 protein were determined by Western blot analysis. (B) Following scanning densitometry of cyclin D3 band intensities, values from cells not treated with cycloheximide were set at 100%, and other values were expressed as a percentage of these.

analysis. In the untreated control cells, the cyclin D3 protein half-life was about 4.5 h (Fig. 5A). However, the cyclin D3 half-life increased by at least 10-fold in response to calpain inhibitor LLnL (Fig. 5).

Discussion

The regulation of cell cycle progression is a complex process which involves proteases action, kinase cascades, production of second messagers and other operations. Increasing evidence now compellingly suggests that changes in the intracellular calcium concentration may also have a crucial role. The non lysosomal and cytosolic Ca^{2+}-dependent neutral proteases calpains are considered to be important regulators of signal transduction pathway[21,27]. They have been shown to be ubiquitously distributed in cells of multicellular animals and belong to the family of cystein proteases. Calpains have not been found in unicellular organisms, and they have been postulated to have a role in the social regulation of growth of animal cells[20,27,33]. These exist in several forms, the most prevalent being μ-calpain (calpain I) and m-calpain (calpain II), which have similar biochemical features in substrate specificity but differ in their *in vitro* requirements of calcium for maximal activity[18,26,33]. μ-Calpain is activated at micromolar concentration of Ca^{2+} while m-calpain requires millimolar Ca^{2+}concentrations, although these calcium requirements may be lowered by autolysis and various modulatory factors[19]. Each enzyme is a heterodimer consisting of an 80 kDa catalytic subunit, unique to each isozyme and encoded by a separate gene, and a smaller 30 kDa regulatory subunit which is identified in all isoforms[29]. Both subunits can bind calcium, but in addition calpain activity can also be regulated by autoproteolysis and the specific endogenous protein inhibitor calpastatin[21,27], suggesting that, like the proteasome, calpains are part of a regulatory proteolytic system. The balance between calpain and calpastatin might determine wether the protein can be targeted for degradation. The exact functions of calpains are still obscure, but a role of calpains in an array of cellular and pathological processes, including platelet aggregation, neuronal long-term poteniation, neutrophill activation, oocyte maturation, differentiation, ischemic cellular injury and apoptosis has been suggested[24,26,30,31]. At present the physiological functions of calpains remain unclear, mainly due to the fact that many physiological substrates have not yet been definitively identified. To date, several transcription factors, such as c-jun, c-fos, and tumor suppressor gene products, including p53 and pRB, were found to be cleavaged by calpain to produce specific partial digestion products[8,10,15,17,34].

Cyclin proteolysis is essential for cell cycle progression. The molecular mechanisms underlying cyclin periodicity involve both transcriptional and posttranslational regulation. Many cyclin mRNAs exhibit cell cycle-dependent fluctuation, and in some cases, specific transcription factors have been implicated in controlling this periodicity[4,9,25]. Another major determinant of cyclin periodicity is protein stability. Cyclin B1 was one of the first proteins to be identified as a substrate for ubiquitin-targeted degradation [6,13]. Cyclin A and cyclin E have also been shown to be substrates for ubiquitination and proteasome-mediated proteolysis[2,14,22]. In the previous study[3], we have shown that cyclin D1 levels are regulated at the posttranscriprional level by Ca^{2+}-activated protease calpain. In this study, we have tried to demonstrate the calpain-mediated proteolysis of cyclin D3. Treatment with lovastatin and actinomycin D in MDA-MB-231 human breast carcinoma cells resulted in loss of cyclin D3 protein that was completely reversible by incubation of the cells with the calpain inhibitor I LLnL, and not the serine protease inhibitor PMSF (Fig. 1). We investigated the effect of the calpain inhibitor II LLM, which is generally not considered to inhibit the proteasome, as well as the lososomal inhibitors and the caspase inhibitors on cyclin D3 expression. Furthermore, these protease inhibitors did not block the lovastatin and actinomycin D-induced loss of cyclin D3 protein in MDA-MB-231 cells (Fig. 3). LLnL is also reported to block activity of the 26S proteasome. Therefore we tested for ubiquitin- and proteasome-associated proteolysis of cyclin D3. We found that the lactacystin, a specific 26S proteasome inhibitor, completely reversed loss of the cyclin B1 by lovastatin and actinomycin D, which has been shown to be degraded by the proteasome[6] but had no effect on cyclin D3 (Fig. 2). These data suggested that degradation of cyclin D3 protein was through calpain, rather than the proteasome. We next investigated whether inhibition of calpain in growing cells can stabilize cyclin D3 protein. The cyclin D3 protein half-life increased by at least 10-fold in this study in response to calpain inhibitor LLnL (Fig. 5). These data further indicate that the proteolysis of cyclin D3 in these cell cultures is specially calpain-associated.

In vitro degradation experiments revealed that cyclin D3 is a substrate for Ca^{2+}-dependent calpain-mediated proteolysis (Fig. 4). These results indicated that degradation of cyclin D3 protein was through calpain.

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초록 : 인체 유방암세포에서 calpain protease에 의한 cyclin D3의 발현 조절

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Ca^{2+}-농도 의존적으로 활성화되는 neutral protease calpain에 의한 단백질 분해는 세포의 성장을 조절하는데 중요한 단백질들의 역할에 매우 중요한 역할을 한다. Cyclin의 분해는 세포주기의 진행을 위한 필연적인 과정이다. D-type cyclins는 외부자극이나 신호에 의하여 세포주기의 G1 초기에 합성이 된 후 cyclin-dependent kinases (cdk4 및 cdk6)와의 결합하여 세포주기 S기 진입을 촉진하는 역할을 한다. 본 연구에서는 MDA-MB-231 인체 유방암세포에서 cyclin D3 단백질이 calpain protease에 의하여 전사 후 수준에서 조절 받고 있음을 제시하였다. 본 실험의 조건에서 lovastatin과 actinomycin D가 처리된 MDA-MB-231 세포에서 cyclin D3 단백질의 발현이 완전히 사라졌지만, calpain inhibitor인 LLnL의 처리에 의하여 정상 수준으로 회복되었음을 알 수 있었다. 그러나 26S proteasome의 선택적 억제제인 lactacystin, the lysosome 억제제인 ammonium chloride 및 chloroquine, serine protease 억제제인 PMSF는 동일 조건에서 lovastatin과 actinomycin D 처리에 의한 cyclin D3의 발현저하를 억제하지는 못하였다. *In vitro* 조건에서 순수 분리된 calpain은 cyclin D3 단백질을 Ca^{2+} 농도 의존적으로 분해하였으며, cyclin D3 단백질의 half-life는 LLnL 처리에 의하여 매우 유의적으로 증가되었다. 이러한 결과는 cyclin D3 단백질이 Ca^{2+}에 의해 활성화 되는 protease calpain에 의해 조절됨을 보여준다.